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Periodic Activation of Wnt/β-Catenin Signaling Enhances Somatic Cell Reprogramming Mediated by Cell Fusion

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SUMMARY

Reprogramming of nuclei allows the dedifferentiation of differentiated cells. Somatic cells can undergo epigenetic modifications and reprogramming through their fusion with embryonic stem cells (ESCs) or after overexpression of a specific blend of ESC transcription factor-encoding genes. We show here that cyclic activation of Wnt/β-catenin signaling in ESCs with Wnt3a or the glycogen synthase kinase-3 (GSK-3) inhibitor 6-bromoindirubin-3′-oxime (BIO) strikingly enhances the ability of ESCs to reprogram somatic cells after fusion. In addition, we show that reprogramming is triggered by a dose-dependent accumulation of active β-catenin. Reprogrammed clones express ESC-specific genes, lose somatic differentiation markers, become demethylated on *Oct4* and *Nanog* CpG islands, and can differentiate into cardiomyocytes in vitro and generate teratomas in vivo. Our data thus demonstrate that in ESCs, periodic β-catenin accumulation via the Wnt/β-catenin pathway provides a specific threshold that leads to the reprogramming of somatic cells after fusion.

INTRODUCTION

The Wnt/β-catenin signaling pathway regulates a variety of cellular processes during the development of vertebrates and invertebrates, including cell proliferation and differentiation, cell fate, and organogenesis (Karner et al., 2006; Reya and Clevers, 2005). In addition, the Wnt/β-catenin pathway controls tissue homeostasis and regeneration in response to damage in zebrafish, *Xenopus*, planarians, and even adult mammals (Gurley et al., 2008; Ito et al., 2007; Osakada et al., 2007; Petersen and Reddien, 2008; Stoick-Cooper et al., 2007; Yokoyama et al., 2007).

β-catenin links cadherins to the cytoskeleton in cell-cell adhesion and acts as the intracellular signaling molecule of the Wnt pathway. The binding of Wnt ligands to Frizzled and LRP5/6 receptors results in inactivation of a multiprotein destruction complex, which is composed of glycogen synthase kinase-3 (GSK-3), Axin, and adenomatous polyposis coli (APC). This inactivation leads to a decrease in β-catenin phosphorylation, which then allows β-catenin to escape ubiquitin-proteasome-mediated degradation (Willert and Jones, 2006). As a consequence, nondegraded β-catenin accumulates in the cytoplasm and translocates into the nucleus, leading to transcriptional activation of a plethora of target genes that control a variety of cellular and tissue processes (Hoppler and Kavanagh, 2007). In the absence of Wnt activation, β-catenin is phosphorylated by the destruction complex and degraded by the ubiquitin-proteasome system. Activation of Wnt/β-catenin signaling induces the expression of multiple antagonists that act in a coordinated manner to shut down the pathway at many levels simultaneously. Extracellularly, the Wif-1 and Dkk proteins inhibit Wnt signaling by interacting with the Wnt protein and LRP5/6 receptors, respectively (Kawano and Kypta, 2003). Intracellularly, both Nkd-1 and Axin2 enhance β-catenin degradation through an increase in the formation of the destruction complex (Behrens, 2005; Wharton et al., 2001).

The Wnt proteins form a large family of isoforms (from Wnt1 to Wnt10b, Wnt11, and Wnt16) (Kikuchi et al., 2007). Among these, Wnt3a has been shown to enhance self-renewal and to maintain totipotency of embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs) (Anton et al., 2007; Reya et al., 2003) through the accumulation of β-catenin in the cell nucleus. Likewise, both inactivation of the *GSK-3α* and *GSK-3β* homologs and mutations in *APC* lead to β-catenin stabilization and severely compromise the ability of ESCs to differentiate and to maintain their stemness (Doble et al., 2007; Kielman et al., 2002; Sato et al., 2004).

Reprogramming can allow dedifferentiation of differentiated cells to pluripotency and can take place via different mechanisms. Somatic nuclear transfer has allowed the cloning of different animals through the reprogramming of a differentiated nucleus (Vajta, 2007). Recent findings have shown that overexpression of four specific ESC transcription factors encoding genes in mouse and human somatic cells can induce them to become pluripotent (Aoi et al., 2008; Brambrink et al., 2008; Hanna et al., 2008; Kim et al., 2008; Lowry et al., 2008; Maherali et al., 2007; Park et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). Somatic mouse and human cells can also be reprogrammed by fusion with ESCs and Nanog, a factor that maintains ESCs in their undifferentiated state and augments the pluripotency of the hybrids (Cowan et al., 2005; Silva et al., 2006; Tada et al., 1997, 2001). In ESC-thymocyte hybrids, reactivation of the inactive X chromosome, erasure of DNA methylation associated with imprinted genes, and reactivation of the Oct4-GFP transgene have all been seen. Moreover, when these hybrid cells were introduced into host diploid blastocysts, they contributed to the three germ layers of the chimeric embryos (Tada et al., 1997, 2001). Similar

findings have been reported after the fusion of human fibroblasts with human ESCs. The tetraploid hybrids obtained showed the phenotype and growth rate of the human ESCs. The somatic genome was reprogrammed, as demonstrated by human-ESC-like genomewide transcriptional analysis (Cowan et al., 2005).

Since Wnt/β-catenin signaling controls ES self-renewal and the maintenance of stemness (Sato et al., 2004) and regulates the expression of ESC genes (Cole et al., 2008), we hypothesized that the Wnt/β-catenin signaling pathway can also control the reprogramming of somatic cells to pluripotency. Here we show that periodic β-catenin accumulation enhances reprogramming of somatic cells via cell fusion. By culturing ESCs transiently with Wnt3a or with the GSK-3 inhibitor 6-bromoindirubin-3′-oxime (BIO), we show that cyclic Wnt/β-catenin signaling enables these treated ESCs to reprogram a variety of different somatic cells after fusion. The isolated reprogrammed clones are pluripotent, since they are able to differentiate in vitro and in vivo.

RESULTS

Activation of the Wnt/β-Catenin Signaling Pathway Enhances Reprogramming of Somatic Cells after Fusion

We used polyethyleneglycol (PEG) to generate fusion hybrids between ESCs constitutively expressing the *NeoR* gene, and mouse neural stem (NS)-Oct4-puro/GFP cells (Silva et al., 2006) expressing the *PuroR* gene and green fluorescent protein (GFP) under the control of the *Oct4* (*Pou5f1*) regulatory element that is only active in pluripotent cells (Yeom et al., 1996). Immediately after fusion, the cells were cultured in gelatin (without feeders that express different Wnt isoforms [Dravid et al., 2005; Wiese et al., 2006]) for 12, 24, 48, and 72 hr (Figure 1A) in the presence of an optimized concentration of Wnt3a (100 ng/ml; see Figure S1 available online). Hybrids were selected in ESC medium supplemented with puromycin; under these culture conditions, only reprogrammed clones can grow. ESC medium induces differentiation and growth arrest of NS cells and thus of nonreprogrammed hybrids, too. Furthermore, puromycin provides additional selection for the reprogrammed clones (Figure S2).

The resistant colonies were stained for the expression of alkaline phosphatase (AP), an ESC marker, and counted (Figure 1A). These cells had been reprogrammed, as they retained a rounded ESC-like phenotype and expressed AP and Oct4-puro/GFP (Figures 1A and 1B). We selected up to 20-fold more reprogrammed clones with respect to the control after 24 hr of Wnt3a culturing. The number of reprogrammed clones decreased after 72 hr of treatment, indicating that prolonged Wnt3a culturing might diminish the reprogramming efficiency. These data clearly showed that time-dependent Wnt3a treatment greatly enhances the ability of ESCs to reprogram NS cells.

In support of these data, a very high number of AP-positive reprogrammed clones (between 30 and 300) showing an ESC-like phenotype were obtained when mouse embryonic fibroblasts (MEFs) expressing different Wnt isoforms (Dravid et al., 2005; Wiese et al., 2006) were fused with ESCs. This number increased up to 5-fold when these hybrids were cultured in the presence of Wnt3a for 48 hr (Figure 1C). Finally, to confirm that activation of the Wnt/β-catenin pathway enhances somatic cell reprogramming, we inhibited this pathway with Dkk1, an LRP6 receptor inhibitor (Logan and Nusse, 2004). The striking decrease in the number of reprogrammed, AP-positive clones (up to 6-fold; Figure 1C) demonstrates that the Wnt/β-catenin signaling pathway stimulates the reprogramming of somatic cells.

Transient Inhibition of GSK-3 in ESCs Enhances Their Ability to Reprogram Different Somatic Cells

To confirm that cell reprogramming is augmented through the activation of the canonical Wnt/β-catenin signaling pathway, we decided to transiently inhibit GSK-3 by addition to the culture medium of the selective GSK-3 inhibitor 6-bromoindirubin-3′-oxime (BIO) (Meijer et al., 2003). This inhibition of GSK-3 mimics the activation of the pathway via Wnt3a, as it promotes the accumulation of β-catenin in the cell nucleus (Meijer et al., 2003) and maintains the pluripotency of stem cells (Sato et al., 2004). Initially, to exclude a direct role of BIO in enhancing clonogenicity and/or viability of ESCs and hybrid clones, we plated scaled numbers of ESCs or hybrids in BIO-containing medium. This demonstrated that the clonogenicity and viability of both ES and hybrids cultured in the absence and presence of BIO, respectively, were equal (Figures S3A and S3B). In addition, since the Wnt/β-catenin signaling pathway is regulated by an inhibitory feedback loop (Ueno et al., 2007), we tested different concentrations of BIO to determine the concentration that mimics this physiological regulation most closely: addition of 1 μM BIO led to the relatively limited activation of the TOPflash reporter gene (a vector harboring β-catenin binding sites fused to luciferase) and resembled the level of activity upon Wnt3a stimulation (Figure S4A). In addition, higher BIO concentrations were slightly toxic to NS cells (data not shown). Thus, we used 1 μM BIO as standard under the remaining experimental conditions.

We then analyzed the effects of BIO treatment over time. PEG-fused ES-neo × NS-Oct4-puro cells were cultured for 12, 24, 48, and 72 hr with this optimized concentration of BIO (1 μM; Figure 2A). After 24 hr of treatment, we obtained up to 70-fold more reprogrammed clones (from 200 to 1500 total clones), with respect to the control (Figure 2A). Reprogramming was dependent on the time of BIO stimulation, with a decreased number of reprogrammed clones after 72 hr of treatment. The selected hybrids were reprogrammed, as they showed an ESC-like phenotype, were AP positive, and expressed GFP-puro (Figures 2A and 2B). However, to exclude a role of BIO as an enhancer of cell fusion, we performed further control experiments. BIO did not enhance PEG-mediated fusion of ESCs, as there was only a slight increase in the number of clones after the fusion of BIO-pretreated ES-neo cells with ES-hygro cells (Figure S5A). In the fusion of ES × ESCs, we could only select for hybrids and not for reprogrammed clones, and the number of clones obtained was comparable. Furthermore, to better exclude the possibility that BIO has a fusogenic activity on different cell types, we PEG fused ES-neo × NS-Oct4-puro cells for 3 hr and then replated the hybrids at a dilution such that the cells were not in contact with each other. Thus, BIO was added to the cell medium for 12, 24, 48, and 72 hr (Figure S5B). The hybrids were formed before the addition of BIO, and they could not increase further since the cells were not touching each other. Nevertheless, however, reprogrammed clones were selected with a pick at 24 hr of BIO treatment. (Figure S5B). This further

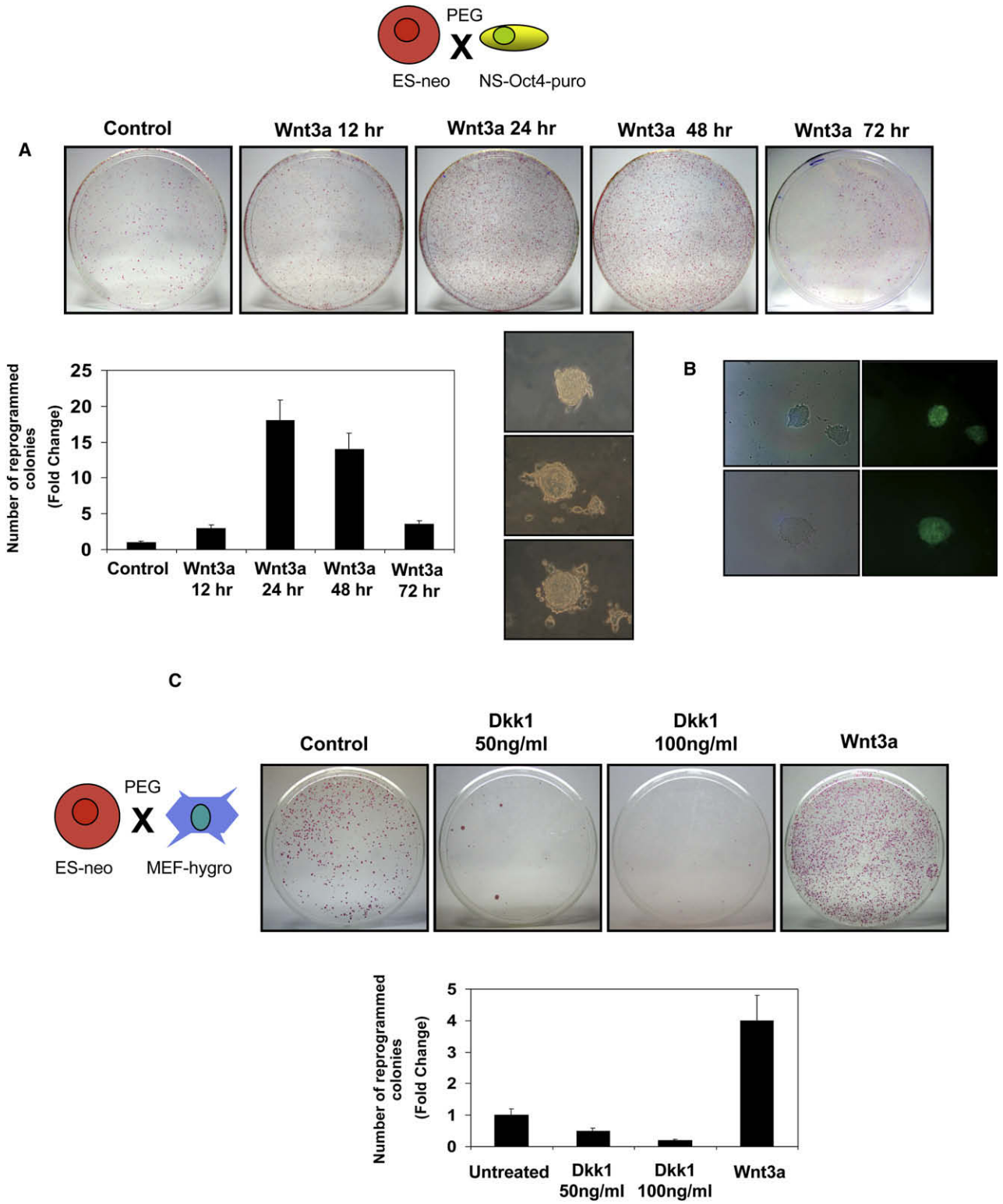


Figure 1. Activation of the Wnt Pathway by Wnt3a Enhances Reprogramming Mediated by Cell Fusion

(A) Representative plates and quantification (as fold increases in the number of colonies) of hybrid colonies from PEG fusions of ES-neo cells with NS-Oct4-puro cells treated with Wnt3a, as indicated. Colonies were stained for expression of AP and counted (mean ± SEM; n = 3).

(B) Fluorescence images of some reprogrammed hybrid colonies following 24 hr Wnt3a treatment (10× magnification).

(C) Quantification of hybrid colonies from PEG fusions of ES-neo cells and MEF-hygro cells untreated and treated with Dkk1 and Wnt3a, as indicated (mean ± SEM; n = 3).

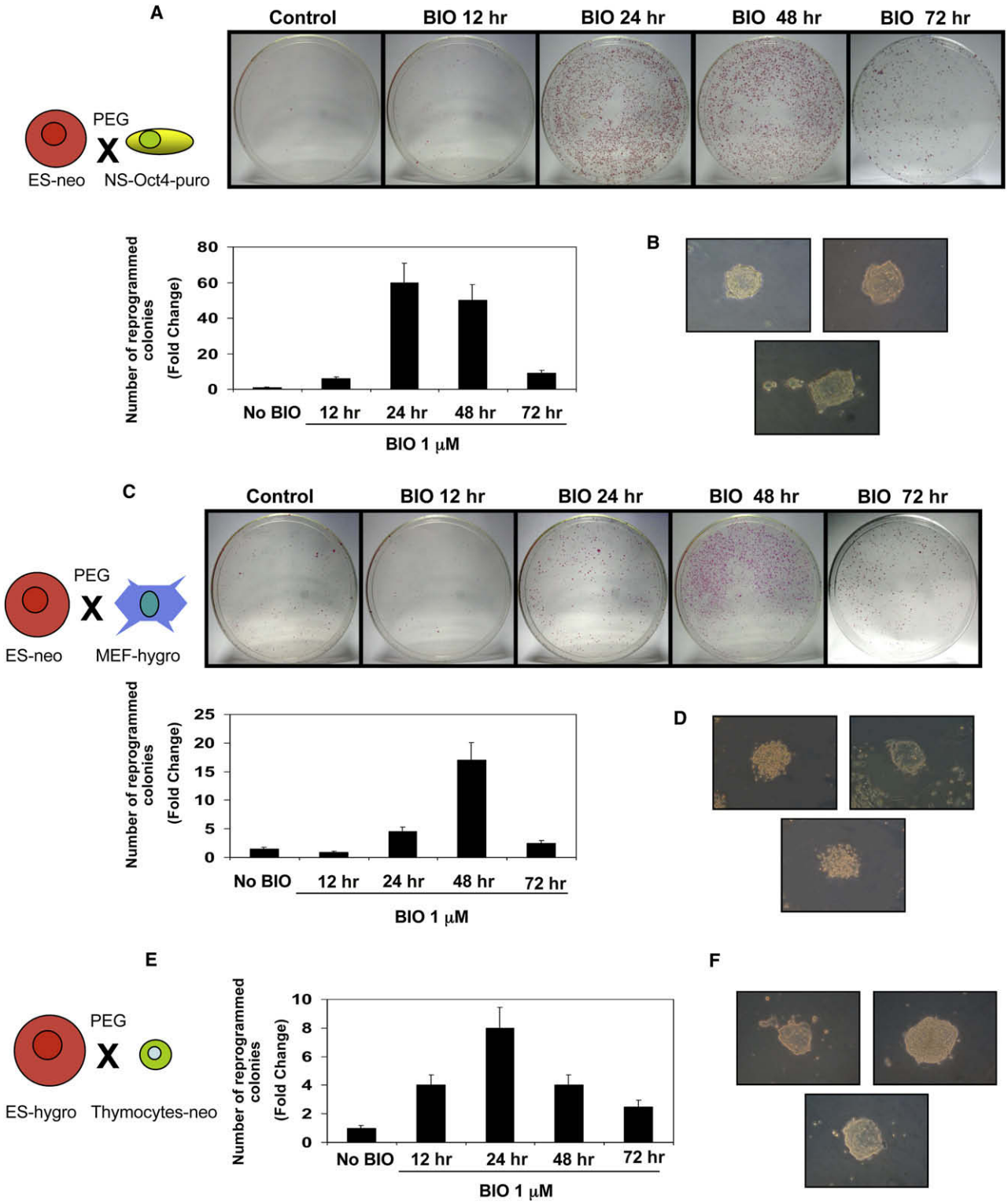


Figure 2. Activation of the Wnt/β-Catenin Pathway by BIO Enhances the Number of Reprogrammed Hybrid Colonies in Three Different Cell-Type Fusions

(A) Quantification (as fold increases in the number of colonies) of hybrid colonies from PEG fusions of ES-neo cells with NS-Oct4-puro cells treated with BIO, as indicated. (A and C) Representative plates and (A, C, and E) fold increases in the number of colonies of hybrids clones from the indicated fusions treated with BIO, as indicated, stained for AP expression and counted. The fold change increases in the reprogrammed colonies are also shown in the graphs (mean ± SEM; n = 3). (B, D, and F) Light microscopy images of some reprogrammed hybrid colonies following 24 hr BIO treatment (10× magnification).

confirms that BIO enhances the reprogramming, and not the fusion, of cells.

Inhibition of GSK-3 with BIO also strikingly increased the numbers of reprogrammed colonies when MEFs and thymocytes were fused with ESCs. PEG-fused hybrids of ES-neo × MEF-hygro and ES-hygro × thymocyte-neo cells were cultured in BIO for 12, 24, 48, and 72 hr and double-drug selected. There were up to 20-fold (between 200 and 1500 total clones) and 9-fold (between 20 and 100 total clones) increases, respectively, in the numbers of reprogrammed selected clones, with respect to their controls (fusions in the absence of BIO; Figures 2C and 2E). Again, the reprogramming efficiency was lower with increased treatment time with BIO. All of the selected clones showed an ES-like phenotype and were AP positive (Figures 2C–2F).

These data demonstrate that transient inhibition of GSK-3 greatly enhances the ability of ESCs to reprogram somatic cells. Furthermore, this reprogramming occurs with variable timing across different cell types.

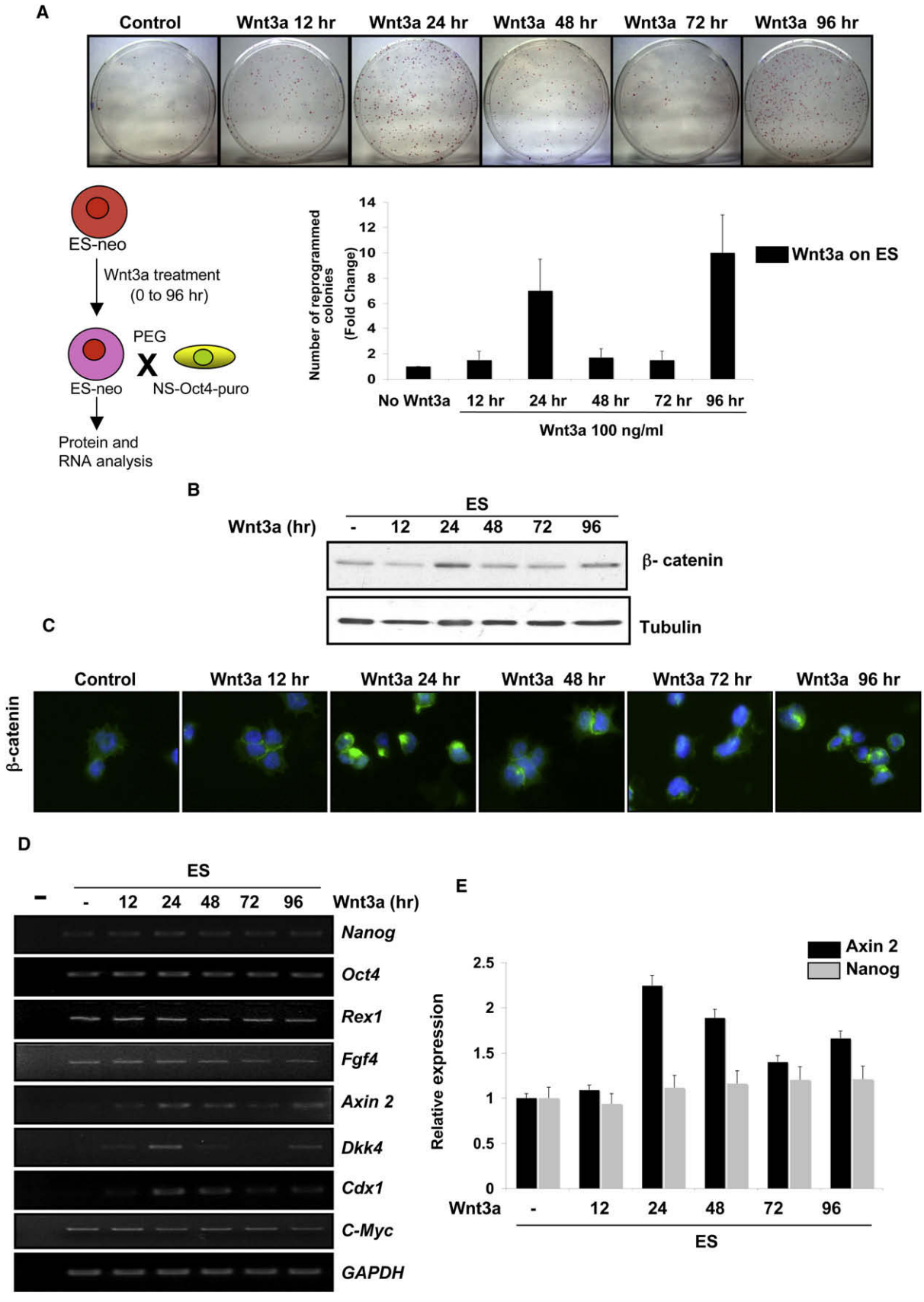
Cyclic Stabilization of β-Catenin in ESCs Enhances Their Ability to Reprogram NS Cells

We then asked whether the activation of the Wnt/β-catenin pathway in ESCs would strengthen and enhance their reprogramming ability. Thus, we investigated whether an enhancement of reprogramming occurs when ESCs and/or NS cells are cultured with Wnt3a or BIO before their fusion. ESCs were thus cultured in Wnt3a or BIO for 12, 24, 48, 72, and 96 hr and fused with NS-Oct4-puro cells grown in the absence of BIO. Up to 15-fold (about 600 total clones) or 80-fold (about 2000 total clones) increases in the numbers of reprogrammed clones were seen when ESCs were precultured for 24 and 96 hr with Wnt3a or BIO, respectively (Figures 3A and 4A); the selected clones showed an ESC-like phenotype, were AP positive, and expressed *Oct4* (Figures 3A, 4A, and 4B). In contrast, there was poor enhancement of NS reprogramming with the ESCs cultured with Wnt3a or BIO for 12, 48, and 72 hr. To ensure that pretreatment of ESCs with BIO before fusing them with NS-Oct4-puro cells did not increase fusion, PEG-fused cells were cultured for 3 hr, an insufficient time for reprogramming to occur, and FACS analysis was carried out. The same number of hybrid clones was seen whether the ESCs were pretreated or not with BIO before the fusion, thus excluding BIO-dependent, enhanced fusogenic activity (Figure S6). We thus asked why there was a parabolic wave of reprogramming through the periods of Wnt3a and BIO treatment. This appeared to be due to β-catenin accumulation in both Wnt3a- and BIO-treated cells at 24 and 96 hr (Figures 3B and 4C) and to its consequent localization in the cell nucleus at these two times of treatment (Figures 3C and 4D). These data show that reprogramming can be triggered only after the cyclic accumulation of β-catenin. While 12 hr of Wnt3a or BIO treatment was not sufficient to accumulate enough β-catenin to trigger reprogramming, 24 hr (and 96 hr) of Wnt3a or BIO pretreatment of ESCs resulted in the accumulation of β-catenin; at the same times, *Axin2*, *Dkk4*, and APC were also expressed, leading to a negative feedback loop on the Wnt/β-catenin pathway (Bazzi et al., 2007; Doble et al., 2007; Jho et al., 2002; Lustig et al., 2002; Niida et al., 2004; Yan et al., 2001) that affected the subsequent 48 and 72 hr time points (Figures 3B, 3D, 3E, 4C, 4E, and 4F and data not shown). The wave of β-catenin accumulation is similar in both Wnt3a- and BIO-treated ESCs. This might be due to the relatively low concentration of BIO used here, which might not inhibit GSK-3 activity fully, thus resembling the feedback loop regulation in Wnt3a-treated cells. Indeed, with 1 μM BIO, the amount of phospho-β-catenin in ESCs was higher with respect to the cells treated with increased BIO concentrations, and moreover, the nuclear accumulation of the nonphosphorylated form was rather low. This leads to the conclusion that GSK-3 was not fully inhibited by 1 μM BIO (Figures S4B and S4C). To confirm that the increased expression of Axin2 was preventing the reprogramming, we generated stable ES clones overexpressing Axin2 (ES-Axin2) and fused them with NS-Oct4-puro cells. No reprogrammed clones were selected after fusion with five different ES-Axin2 clones (Figure S7). In conclusion, activation of Wnt/β-catenin signaling is a key mechanism for the reprogramming of somatic cells, and only when β-catenin accumulates in ESCs to a certain threshold can these cells reprogram NS cells after fusion.

Remarkably, Wnt3a- and BIO-treated ESCs expressed the same levels of the pluripotent genes *Nanog*, *Oct4*, *Rex1*, and *Fgf4* at all of the treatment times, whereas expression of the β-catenin-dependent genes *Cdx1*, *Axin2*, and *Dkk4* cycled according to the accumulation of the β-catenin protein itself (Figures 3D, 3E, 4E, and 4F). Of note, although *c-Myc*, a known β-catenin target gene (He et al., 1998), has previously been shown to enhance reprogramming when overexpressed together with three other genes (Takahashi and Yamanaka, 2006), here it was constantly expressed under Wnt3a and BIO treatments. These data suggest that pretreatment of ESCs with Wnt3a or BIO results in the activation of the Wnt/β-catenin signaling pathway and expression of specific β-catenin target gene(s) that in turn stimulate the reprogramming of somatic cells. This pathway is independent of *Nanog*, *Oct4*, *Rex1*, *Fgf4*, and *c-Myc*; indeed, these genes were expressed at the same levels throughout the different Wnt3a and BIO treatments.

The alternative of pretreatment of NS cells with BIO led to β-catenin accumulation at 24 and 72 hr but provided poor enhancement of reprogramming, with only a slight increase in the number of ESC-like, AP-positive reprogrammed clones (Figure 4A and Figure S8). Thus, the Wnt/β-catenin signaling pathway drives a remarkable enhancement of reprogramming of somatic cells only when it is activated in ESCs.

Next, we wanted to better characterize the mechanism of activation of reprogramming and to ensure that β-catenin is a key factor in the reprogramming mechanism of somatic cells. We asked whether ESCs expressing different levels of β-catenin have different reprogramming abilities. For this, we generated stable ES clones overexpressing different levels of the stabilized β-catenin (ES-β-catenin) harboring a mutation in one of its destruction-complex-dependent phosphorylation sites. We selected five clones that activated expression of the TOPflash reporter gene with different degrees of activity, with E1 and F19 being the least active, A13 and A12 with intermediate activity, and C2 being the most active and the most similar to *GSK-3*$^{-/-}$ ESCs, which accumulate a constant and high amount of β-catenin (Doble et al., 2007) and greatly activate TOPflash (Figure 5A). A very high number of reprogrammed clones (up to a 55-fold increase) was generated after fusion of the E1 and of


A
Control
Wnt3a 12 hr
Wnt3a 24 hr
Wnt3a 48 hr
Wnt3a 72 hr
Wnt3a 96 hr
ES-neo
Wnt3a treatment
(0 to 96 hr)
PEG
ES-neo
NS-Oct4-puro
Protein and
RNA analysis
Number of reprogrammed colonies (Fold Change)
14
12
10
8
6
4
2
0
Wnt3a on ES
No Wnt3a
12 hr
24 hr
48 hr
72 hr
96 hr
Wnt3a 100 ng/ml
B
ES
Wnt3a (hr)
-
12
24
48
72
96
β- catenin
Tubulin
C
Control
Wnt3a 12 hr
Wnt3a 24 hr
Wnt3a 48 hr
Wnt3a 72 hr
Wnt3a 96 hr
β-catenin
D
ES
-
-
12
24
48
72
96
Wnt3a (hr)
Nanog
Oct4
Rex1
Fgf4
Axin 2
Dkk4
Cdx1
C-Myc
GAPDH
E
Axin 2
Nanog
Relative expression
2.5
2
1.5
1
0.5
0
Wnt3a
-
12
24
48
72
96
ES

F19 clones with NS-Oct4-puro cells, an intermediate number was generated upon fusion with the A13 and A12 clones (up to an 18-fold increase), and almost no reprogrammed clones were selected after fusion of the C2 clone and of *GSK-3*$^{-/-}$ cells (Figure 5B). Of note, the E1 clone activated the TOPflash reporter gene to a similar extent to that seen for 1 μM BIO or Wnt3a 24 hr-treated cells (Figure 5C), leading to the conclusion that these cells express similar levels of active β-catenin and therefore have comparable reprogramming capacities. These data clearly demonstrate that reprogramming occurs only when the levels of β-catenin are neither too low nor too high; the levels of β-catenin need to be within an intermediate physiological level, which the E1 and F19 clones and both Wnt3a- and 1 μM BIO-treated ESCs have.

Isolated Clones Are Reprogrammed and Can Differentiate In Vitro and In Vivo

To confirm pluripotency, and thus the ESC-like phenotype, 21 different reprogrammed clones were isolated from the fusion of ESCs pretreated with BIO for 24 hr with NS-Oct4-puro cells. The clones were all tetraploids (Figure 6A), were GFP positive, and expressed *Oct4*, *Nanog*, and *Rex1* after several passages (Figure 6B). In contrast, the neural *Blbp* and *Olig2* genes were shut off (Figure 6C and Figure S9). Likewise, ES × thymocytes and ES × MEF reprogrammed hybrids expressed *Oct4* and *Nanog* and lost expression of *CD4* and *CD8*, and *Col1a1*- and *Col1a2*-specific markers, respectively (Figure S10A). Furthermore, bisulfite genomic sequence analysis revealed that in the ES × NS reprogrammed clones, the CpG islands of the *Nanog* and *Oct4* promoters were highly demethylated and were comparable to ESCs, suggesting that these two loci had undergone massive epigenetic modifications; in contrast, the same CpGs were highly methylated in parental NS cells (Figure 6D). These data clearly show that the hybrids isolated from the fusions with ESCs pretreated with BIO had been reprogrammed.

Next, to determine the differentiation ability of the reprogrammed clones, we induced those of ES × NS, ES × thymocytes, and ES × MEFs to spontaneous differentiation. Embryoid bodies were formed: ball-shaped structures were clearly visible after 3 days and underwent differentiation after 6 and 9 days (Figure 6E and Figure S10B). Expression of *Oct4* (pluripotent stem cell), *AFP* (endoderm), *Brachyury* (mesoderm), *Nkx2.5* (cardiac muscle), and *GATA4* (cardiac muscle) were seen at 3, 5, 7, and 9 days of differentiation, with their expected timing (Boheler et al., 2002), and as comparable to ESCs (Figure 6F and Figure S10C). Furthermore, some of the clones started beating spontaneously, demonstrating their differentiation into cardiomyocytes (Figure S11).

Finally, to test the differentiation ability of ES × NS hybrids in vivo, we transplanted five different clones subcutaneously in immunodeficient (SCID) mice. Three weeks after injection, teratomas were seen (Figure S12). Histological examination showed the presence of gut-like epithelial tissue (endoderm), striated muscle and adipose tissue (mesoderm), neural tissue, epidermis, and cartilage (ectoderm) (Figure 6G).

These data clearly demonstrate the differentiation ability of the reprogrammed clones, both in vitro and in vivo.

DISCUSSION

We have shown here that transient activation of the Wnt/β-catenin signaling pathway leads to a cyclic accumulation of β-catenin in ESCs that enables them to reprogram somatic cells after fusion. When and only when a specific level of β-catenin is stabilized and translocates into the nucleus are ESCs able to reprogram differentiated cells; in contrast, when β-catenin is degraded, reprogramming does not occur. The different levels of β-catenin over time are likely to be due to the expression of some of its inhibitory targets, such as Axin2 and Dkk4, which stabilize the destruction complex and inhibit Wnt receptors, respectively. A negative feedback loop of the Wnt/β-catenin signaling pathway has been shown in differentiating ESCs, which can result in both positive and negative effects on cardiogenesis (Ueno et al., 2007). Indeed, we have shown that overexpression of Axin2 in ESCs impairs their reprogramming ability. The periodic accumulation of active β-catenin or low amounts of a stabilized β-catenin mutant are thus essential in the reprogramming process; in contrast, stable and high levels of β-catenin affect the ability of ESCs to reprogram somatic cells (Figure 7).

Why is the level and timing of β-catenin accumulation so important for reprogramming efficiency? A stable gain-of-function form of β-catenin can lead to tumor formation (Katoh and Katoh, 2007). In addition, *GSK-3*$^{-/-}$ ESCs that express stable β-catenin levels cannot differentiate (Doble et al., 2007; Kielman et al., 2002), and we have shown that these cells, as ES clones harboring high β-catenin activities, are not able to reprogram somatic cells. On the other hand, a loss of function of β-catenin has severe defects in mouse development at the gastrulation stage (Haegel et al., 1995; Huelsken et al., 2000) and *β-catenin*$^{-/-}$ ESCs do not express some stem cell markers (Anton et al., 2007). Furthermore, we have shown here that basal levels of active β-catenin in ESCs can activate reprogramming only very poorly.

Thus, both loss of function and gain of function of β-catenin are highly toxic for cells, with grave effects on their differentiation ability and capacity to induce reprogramming. In contrast, transient Wnt/β-catenin signaling activation is regulated by the

Figure 3. Cyclic Wnt/β-Catenin Signaling Pathway Activation via Wnt3a Enables ESCs to Reprogram NS Cells without Activation of Stem Cell Genes

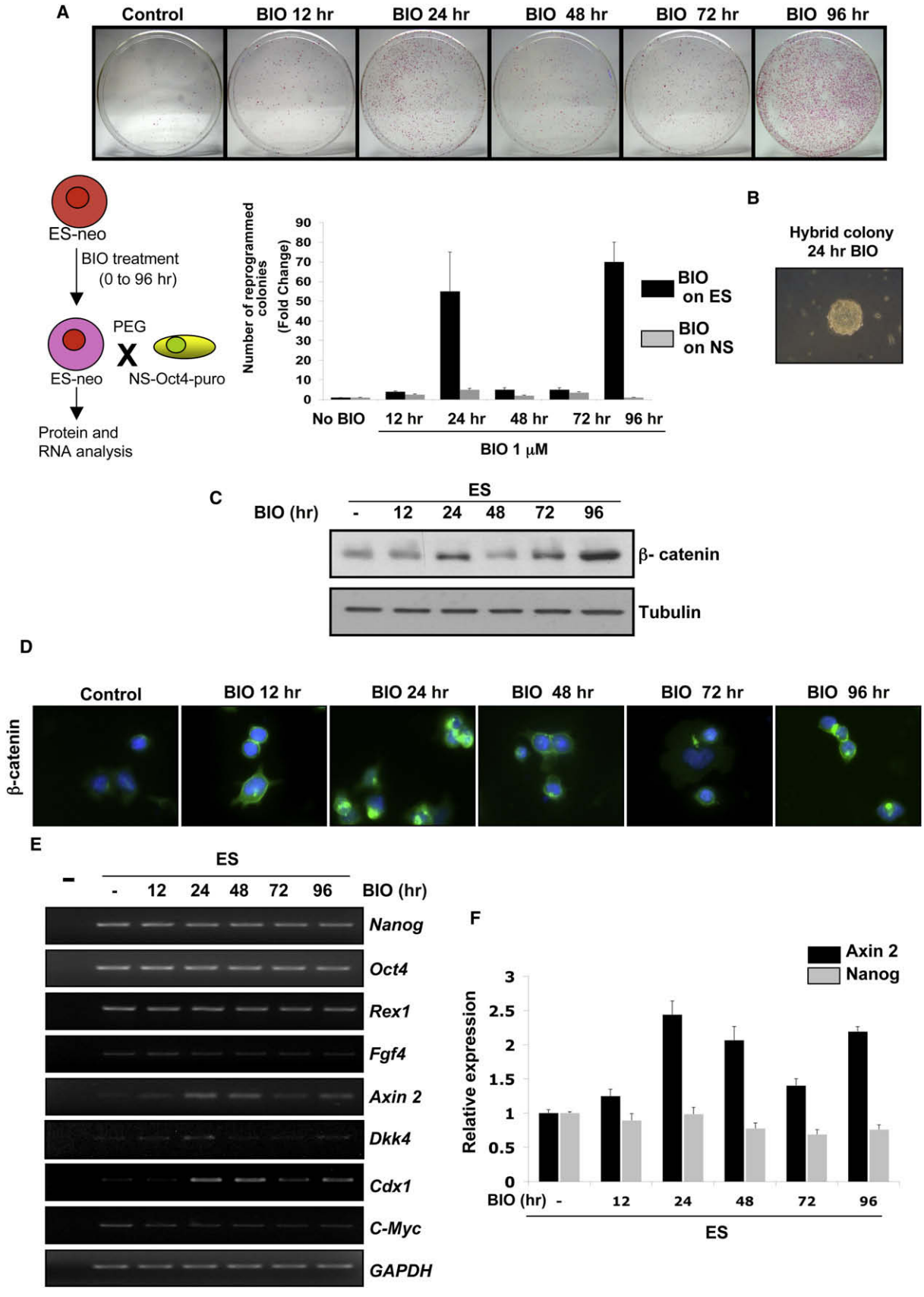
(A) Representative plates and quantification (as fold increases in the number of colonies) of hybrid colonies from PEG fusions between Wnt3a-pretreated ES-Neo cells and nontreated NS-Oct4-puro cells, as indicated. Colonies were stained for the expression of AP and counted (mean ± SEM; n = 3).

(B) Western blotting of protein extracts from ESCs from the Wnt3a-pretreatments in (A), as indicated.

(C) Immunofluorescence for the visualization of β-catenin localization in ESCs from the Wnt3a-pretreatments in (A), as indicated.

(D) Semiquantitative RT-PCR analysis of ESC-specific genes (*Nanog*, *Oct4*, *Rex1*, and *Fgf4*) and β-catenin targets (*Axin2*, *Cdx1*, and *c-Myc*) in total mRNA purified from ESCs from the Wnt3a-pretreatments in (A), as indicated.

(E) Quantitative RT-PCR analysis of *Axin2* and *Nanog* transcript levels in ESCs from the Wnt3a-pretreatments in (A), as indicated (mean ± SEM; n = 3).


A
Control
BIO 12 hr
BIO 24 hr
BIO 48 hr
BIO 72 hr
BIO 96 hr
ES-neo
BIO treatment
(0 to 96 hr)
PEG
X
ES-neo
NS-Oct4-puro
Protein and
RNA analysis
Number of reprogrammed colonies (Fold Change)
90
80
70
60
50
40
30
20
10
0
No BIO
12 hr
24 hr
48 hr
72 hr
96 hr
BIO 1 μM
BIO on ES
BIO on NS
B
Hybrid colony
24 hr BIO
C
ES
BIO (hr)
-
12
24
48
72
96
β- catenin
Tubulin
D
Control
BIO 12 hr
BIO 24 hr
BIO 48 hr
BIO 72 hr
BIO 96 hr
β-catenin
E
ES
-
12
24
48
72
96
BIO (hr)
Nanog
Oct4
Rex1
Fgf4
Axin 2
Dkk4
Cdx1
C-Myc
GAPDH
F
Axin 2
Nanog
Relative expression
3
2.5
2
1.5
1
0.5
0
BIO (hr) -
12
24
48
72
96
ES

negative feedback loop that tunes the level of β-catenin, resulting in waves of its accumulation up to a specific threshold and the induction of reprogramming.

Once in the nucleus, β-catenin activates different target genes (Katoh and Katoh, 2007). Here, we postulate that one or more factors, the "reprogrammers" that are not constitutively expressed in ESCs, are transcribed upon β-catenin binding and switch on a cascade of events that triggers the reprogramming of the genome of the somatic nucleus. This is suggested also by the finding that reprogramming occurs with variable timing across various cell types, which leads to the prediction that β-catenin initiates a complex program of events that can last for different times in different cell types.

Reprogramming could be due to *trans*-acting factors that can trigger transcription and/or suppression of somatic-nucleus-specific genes, implying that these factors must be present constantly to maintain the nondifferentiated phenotype. Alternatively, the induction of a new heritable epigenetic modification of the somatic nucleus could switch a program, resulting in massive chromatin modifications. Whatever the mechanism is, the transcription of the reprogrammer(s) itself is probably finely tuned by checkpoint controls that regulate its concentrations; indeed, high levels of stabilized β-catenin in *GSK-3*$^{-/-}$ or C2 cells that had skipped destruction complex inactivation abolished the reprogramming ability of ESCs, leading us to envisage that there are alternative checkpoint systems that control the concentrations of the reprogrammer(s) that might come into action.

In human and mouse ESCs, the polycomb group (PcG) proteins show transcriptional repression of the expression of developmental genes, which would otherwise promote differentiation (Boyer et al., 2006b; Lee et al., 2006). The chromatin structure of many of these PcG-regulated genes contains "bivalent domain" modifications that consist of both inhibitory histone H3 lysine 27 methylation and activating histone H3 lysine 4 methylation marks (Bernstein et al., 2006). Furthermore, master regulators of pluripotency, like Oct4, Sox2, and Nanog, control genes that encode transcription factors, chromatin-modifying enzymes, and signal-transduction proteins that regulate ESC self-renewal (Boyer et al., 2006a; Loh et al., 2006). During the reprogramming process, one or more reprogrammers, as specific β-catenin targets, might initiate the modifications of the chromatin state of the somatic nucleus, finally resulting in the establishment of pluripotent, ES-like genomic features, containing PcG-regulated bivalent domain modifications and Oct4-, Sox2-, and Nanog-regulated genes.

In culture, the generation of induced pluripotent stem cells (iPS) demonstrates that pluripotency can be obtained by overexpression of defined transcription factors, including stem cell genes; however, this process is not likely to occur in vivo, since factors like Nanog, Oct4, and Sox2 are not expressed in adult tissues or in adult somatic stem cells and since they are dispensable for adult stem cell function (Lengner et al., 2007). On the other hand, overexpression of a blend of these transcription factors is a potent recipe for the generation of novel stem cells in culture that will hopefully be a powerful resource for medical applications. Our data demonstrate that transient and periodic activation of the Wnt/β-catenin signaling pathway without overexpression of any of the known ESC genes can control the reprogramming of somatic cells, mimicking a physiological scenario that might occur in vivo. Remarkably, adult mouse liver cells express high levels of β-catenin, and the efficiency of selection of iPS-hepatocytes appears to be greater with respect to iPS-fibroblasts, even if the number of retroviral integrations of the reprogramming factors is lower in liver cells (Aoi et al., 2008). These findings indicate that Wnt signaling can enhance the generation of iPS cells. This was recently demonstrated by Marson et al. (2008) while the present paper was in revision, as they showed that iPS lines can be isolated with high efficiency from MEFs cells transduced with lentiviruses encoding Oct4, Sox2, and Klf4 and cultured in Wnt3a-CM (Wnt3a-conditioned medium). Of note, and in agreement with our data, Marson et al. (2008) showed that the Wnt-mediated mechanism of reprogramming is independent of c-Myc. However, they were not able to reproduce the effects of Wnt3a-CM on reprogramming with GSK-3 inhibitors. This was potentially due to the prolonged culture in medium containing the inhibitors that, as we have shown here, have negative effects on reprogramming.

Isolated reprogrammed clones can differentiate in vivo and in vitro. The process of achieving full reprogramming is slow, as was seen in iPS cells that expressed detectable amounts of GFP weeks after antibiotic selection (Jaenisch and Young, 2008). Interestingly, in the present study, the selected hybrids were soon fully reprogrammed while they were drug selected, since they expressed high levels of GFP and puromycin at the same time.

One question arises finally: can the Wnt signaling-mediated reprogramming of somatic cells occur upon spontaneous cell fusion? PEG-independent fusion has been shown for different cell types, but it is a very rare event (Ogle et al., 2005). This is probably because the majority of hybrids formed are forced to undergo differentiation under the culturing conditions used. It will be of interest in the future to determine if reprogramming of spontaneously fused cells can be enhanced by the activation of the Wnt/β-catenin signaling pathway. This

Figure 4. Periodic Accumulation of β-Catenin in ESCs via BIO Inhibition of GSK-3 Augments the Reprogramming of NS Cells without Activation of Stem Cell Genes

(A) Representative plates and quantification of hybrid colonies from PEG fusions between BIO-pretreated ES-Neo cells and nontreated NS-Oct4-puro cells, as indicated. Colonies were stained for the expression of AP and counted. The quantification also shows BIO-pretreated NS-Oct4-puro cells fused with nontreated ES-Neo cells (gray bars) (mean ± SEM; n = 3).

(B) Light microscopy image of a reprogrammed hybrid colony isolated from fusions with 24 hr BIO-pretreated (24 hr) ES-neo cells and NS-Oct4-puro cells (10× magnification).

(C) Western blotting of protein extracts from BIO-pretreated ESCs in (A), as indicated.

(D) Immunofluorescence for the visualization of β-catenin localization in ESCs from the BIO-pretreatments in (A), as indicated.

(E) Semiquantitative RT-PCR analysis of ES markers (*Nanog*, *Oct4*, *Rex1*, and *Fgf4*) and β-catenin targets (*Axin2*, *Cdx1*, and *c-Myc*) in total mRNA purified from BIO-pretreated ESCs in (A), as indicated.

(F) Quantitative RT-PCR analysis of *Axin2* and *Nanog* transcripts in BIO-pretreated ESCs (mean ± SEM; n = 3).

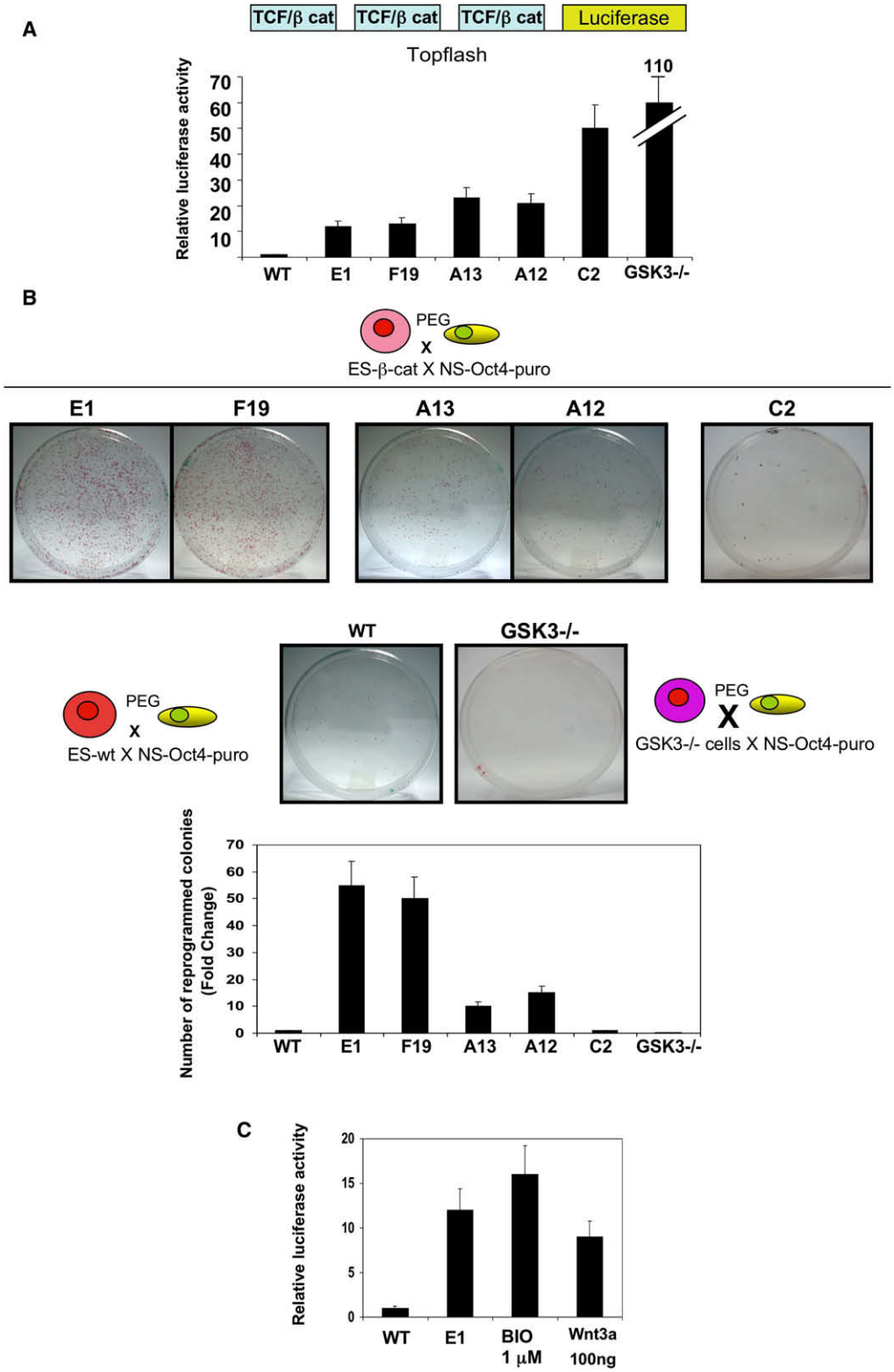


Figure 5. Low Levels of Stabilized β-Catenin Induce Reprogramming of NS Cells

(A) Luciferase reporter assay of ES-β-catenin clones nucleofected with the TOPflash reporter construct.

(B) Quantification (as fold increases in the number of colonies) of hybrid colonies from PEG fusions of ES-β-catenin clones (E1, F19, A13, A12, C2) with NS-Oct4-puro cells, and from PEG fusion of wild-type ES or *GSK-3*$^{-/-}$ cells with NS-Oct4-puro cells, as indicated (mean ± SEM; n = 3).

(C) Luciferase reporter (TOPflash) assay of ESCs untreated and treated for 24 hr with 1 μm BIO or 100 ng Wnt3a and of the E1 clone (mean ± SEM; n = 3).

might represent the starting point to explore whether the reprogramming of spontaneously fused cells can occur in living organisms.

EXPERIMENTAL PROCEDURES

Cells

NS-Oct4-puro cells isolated from HP165 mice and carrying the regulatory sequences of the mouse *Oct4* gene driving GFP and puromycin resistance genes were a gift from Dr. A. Smith; they were cultured as previously described (Conti et al., 2005). Thymocytes were isolated from 6- to 8-week-old mice. Hygromycin-resistant MEFs were purchased at passage 3 (Millipore). ES-neo and ES-hygro cells were derived from E14Tg2a and transduced with the lentiviral pHRcPPT-PGK-neomycin and pHRcPPT-PGK-hygromycin vectors (Zito et al., 2005). ES GSK-3 DKO cells were a gift from Dr. Doble, and they were cultured as previously described (Doble et al., 2007). ESCs were cultured on gelatin in knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Hyclone), 1× nonessential amino acids, 1× GlutaMax, 1× 2-mercaptoethanol, and 1000 U/ml LIF ESGRO (Chemicon).

Cell Hybrids

ES × NS and ES × MEF PEG fusions: 1.2 × 10^6 NS cells or MEFs were plated into T25 flasks. After 2 hr, 1.2 × 10^6 ESCs were plated onto them, allowing them to attach to the NS cells or MEFs. After a further 2 hr, 1 ml 50% (w/v) PEG 1450 (Sigma; prewarmed to 37°C) was added for 2 min, and then the cells were washed three times with serum-free DMEM. Finally, they were cultivated with ESC complete medium without and with 1 μM BIO (Calbiochem), 100 ng/ml Wnt3a (R&D Systems), or 50–100 ng/ml DKK1 (R&D Systems). After 12 hr, the cells were trypsinized and plated into gelatin + laminin-treated p100 dishes. For the ES × NS selection, puromycin was added after 72 hr, while for the ES × MEF selection, hygromycin + neomycin was added after 24 hr.

ES × thymocyte fusions: the cells were fused in suspensions as previously described (Silva et al., 2006). After fusion, they were packed by centrifugation and the supernatant was discarded. The pelleted cells were resuspended in complete ESC medium and plated. The selection was carried out after 24 hr, using hygromycin plus neomycin.

Pretreated-ES × NS PEG Fusions

1 × 10^6 ESCs were plated in p100 plates and treated for 96, 72, 48, 24, and 12 hr with 100 ng/ml Wnt3a or 1 μM BIO. On the last day, the cells were trypsinized, counted, and plated on NS cells for fusions or harvested for RNA and protein analyses. PEG fusions and drug selection were performed as previously described.

In Vitro Differentiation of Hybrid Cells

The differentiation medium for the production of embryoid bodies consisted of ESC maintenance medium with no LIF supplementation. The cells were harvested by trypsinization, counted, and propagated in hanging drops (400 single ESCs/30 μl initial drop) for 2 days before being transferred to 10 cm^2 bacterial dishes. At day 5, the embryoid bodies were transferred onto gelatinized p100 dishes.

Teratoma Production

Cells were trypsinized into single-cell suspensions and resuspended in phosphate-buffered saline to a concentration of 1.5 × 10^7 cells/ml. These cells were injected subcutaneously into the hind limbs of Fox Chase SCID mice using a 25 gauge needle (200 μl). Teratomas were collected after 4 weeks and were fixed, embedded, sectioned, and stained.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, 12 figures, and four movies and can be found with this article online at http://www.cellstemcell.com/supplemental/S1934-5909(08)00415-3.

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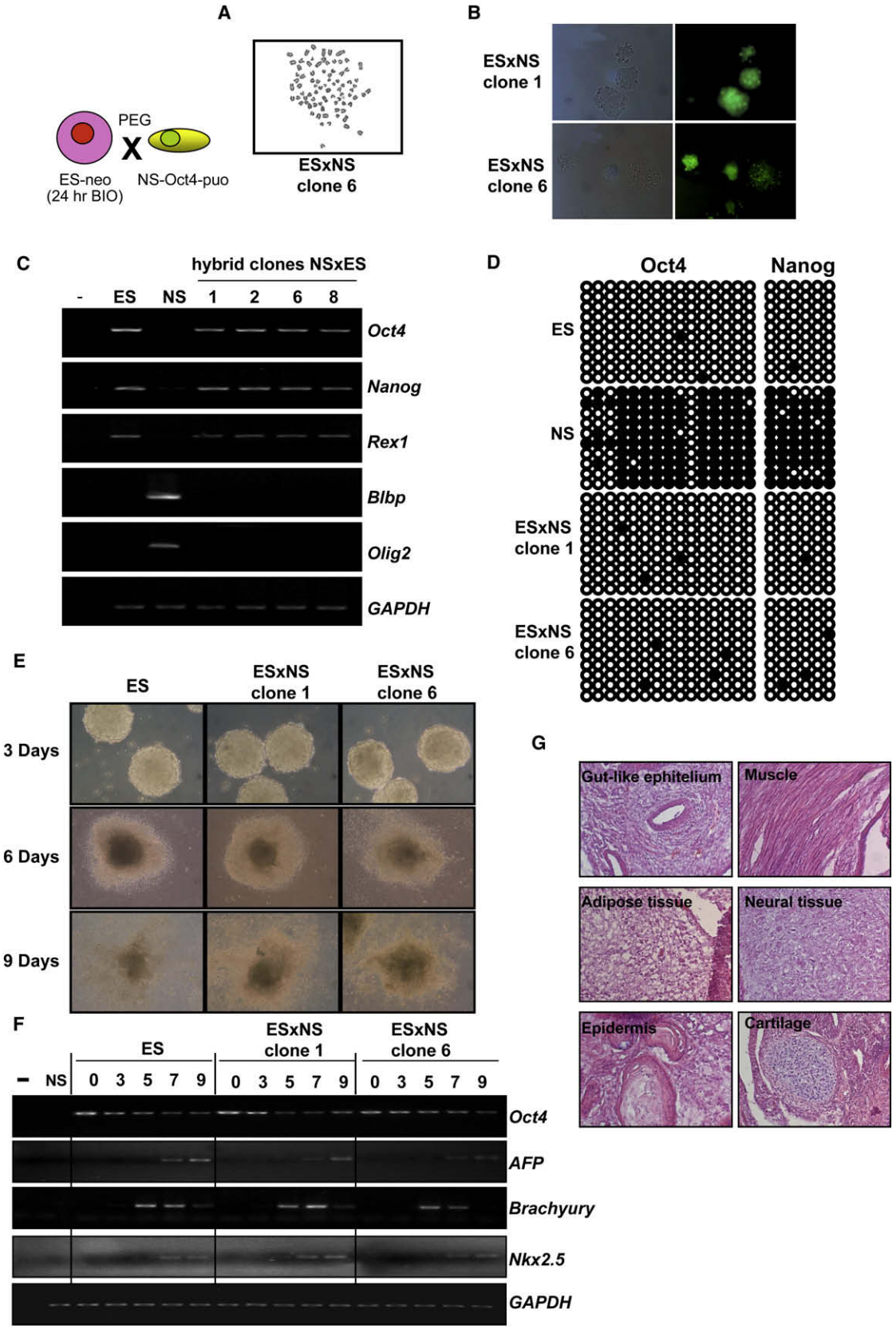
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ES-neo
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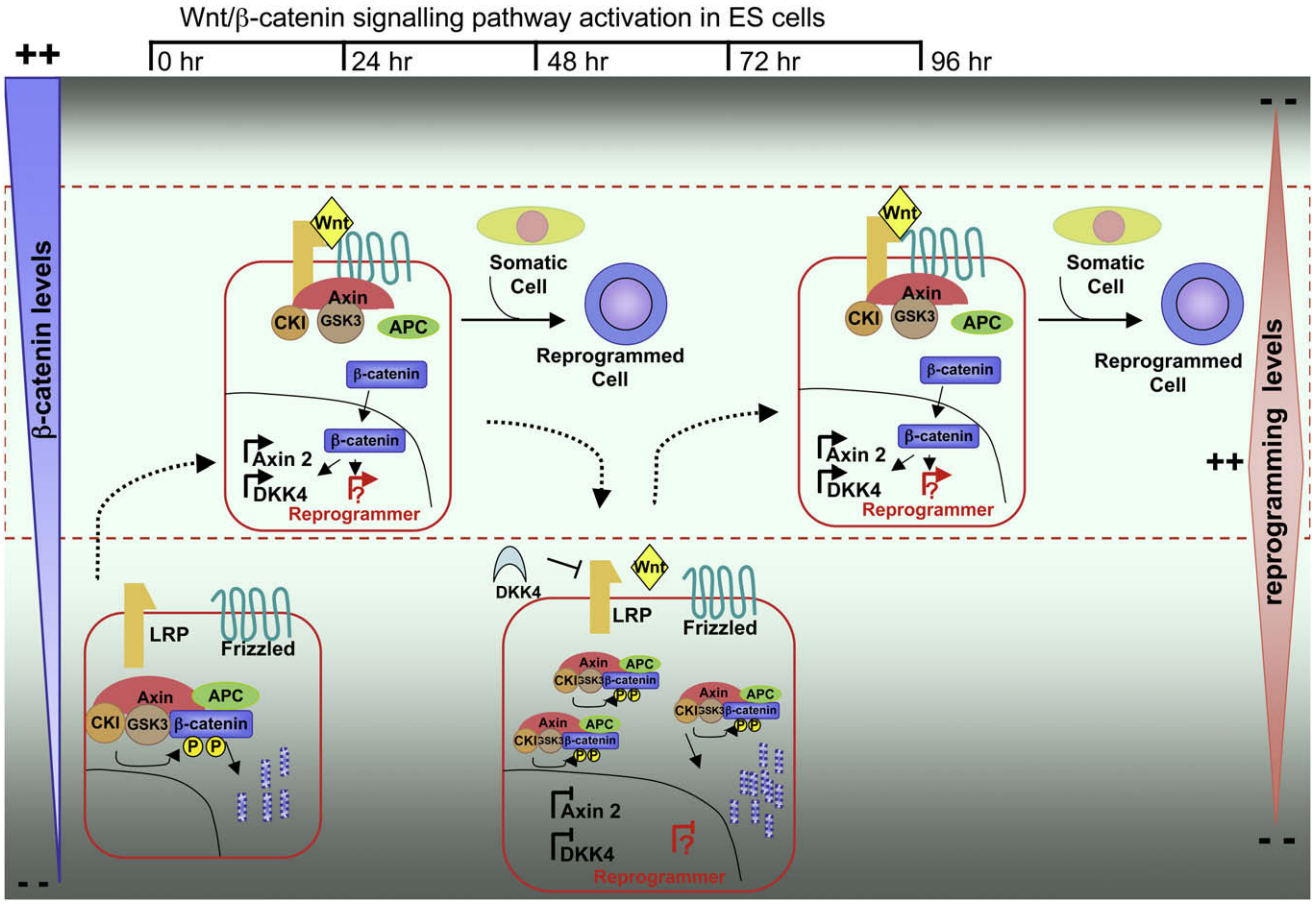

Figure 7. Periodic Accumulation of β-Catenin in ESCs Enables Them to Reprogram Somatic Cells after Fusion

At zero time (nontreated cells), the levels of β-catenin are low due to its degradation via the destruction complex. After 24 hr of Wnt3a or BIO treatment, the Wnt/β-catenin signaling pathway is switched on, and β-catenin is stabilized and enters the nucleus. Once in the nucleus, it activates several target genes, including hypothetic unknown reprogrammer(s), master factor(s) that can drive the reprogramming of somatic cells after fusion. Accumulation of β-catenin also activates transcription of its inhibitors, Axin 2 and Dkk4. In the two subsequent time points, after 48 and 72 hr of Wnt3a or BIO treatment of ESCs, the high levels of Axin 2 increase the formation of the destruction complex and β-catenin is then phosphorylated and degraded. In addition, Dkk4 inhibits Wnt signaling by interacting with its receptor. Thus, ESCs cannot reprogram somatic cells. Finally, 96 hr of Wnt3a or BIO treatment of ESCs leads to activation of Wnt/β-catenin signaling, inactivation of the multiprotein destruction complex, β-catenin accumulation in the nucleus, transcription of the reprogrammer(s), and the consequent ability of the ESCs to reprogram somatic cells after fusion. Very high and constant levels of β-catenin do not allow reprogramming, possibly because checkpoint control mechanisms are activated and act as negative regulators.

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Figure 6. Isolated Hybrids Are Pluripotent and Can Differentiate In Vitro and In Vivo

(A) Normal karyotype and (B) GFP expression in different reprogrammed hybrid clones isolated from fusions of BIO-pretreated (24 hr) ES-neo cells and NS-Oct4-puro cells. (C) RT-PCR analysis of *Oct4*, *Nanog*, *Rex1*, *Blbp*, and *Olig2* in ESCs, NS cells, and some of the hybrid clones, as indicated. (D) Bisulfite genomic sequencing of the promoter regions of *Oct4* and *Nanog* in ESCs, NS cells, and hybrid clones. Open circles indicate nonmethylated CpG dinucleotides; closed circles indicate methylated CpGs. (E) Morphology of embryoid bodies during differentiation, as indicated. (F) RT-PCR analysis of differentiation markers in ESCs, NS cells, and hybrid clones, as indicated. (G) Hematoxylin and eosin staining of teratoma derived from cells of one reprogrammed clone transplanted subcutaneously into SCID mice.

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